Absorption of Chelated Iron by Soybean Roots in Nutrient Solutions [1, 2]

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During the past decade, many investigators have studied the uptake of micronutrients when supplied as chelates to plant roots. Considerable controversy has arisen as to whether the metal is dissociated from the organic ligand at the root membrane and is taken up by itself, or whether the entire chelate is absorbed through the root membrane. Several investigators, notably Wallace and co-workers (1, 7), have offered evidence that the entire chelate is taken up by the plant, whereas others, notably Tiffin and co-workers (3, 4, 5), have offered evidence that the metal is differentially absorbed from the chelate at the root surface.

During the past 18 months the Agricultural Research Center of Stanford Research Institute has been conducting studies on the mineral nutrition of plants with emphasis on iron uptake in soybeans. Certain results were obtained which have a bearing on the general question of absorption of chelated iron through plant roots. These results are reported here in the belief that they may contribute to the answer to this question.

Materials & Methods

Soybean seeds (*Glycine max* L., var's. Hawkeye or PI-54619-5-1) were germinated and grown in sand for 1 week after which time they were transplanted to 1 gallon polyethylene pots containing an aerated nutrient solution (Hoagland No. 2) maintained at pH 6.0. The polyethylene containers were painted on the outside with aluminum paint. Four plants were grown in each pot. Nutrient solutions were made with distilled water and C.P. grade salts. Solutions were brought up to volume every 2 or 3 days. The plants were grown for about five weeks in a complete nutrient solution; at the end of this period they were transferred to solutions containing all minerals except iron. In about two weeks the young leaves of the soybean plants showed typical iron-chlorosis symptoms. At this time the plants were transferred to new nutrient solutions containing various iron treatments, and recovery from the chlorosis was induced. To obtain maximum uniformity of initial chlorosis in the plants, the two plants in each pot which were least uniform were removed before solutions with iron chelates were supplied.

During periods of high light intensity leaves of some plants developed necrotic spots. These leaves were not satisfactory for use in regreening experiments. This condition was alleviated by placing Saran screens transmitting only 69 % of the incident radiation outside the greenhouse during the summer months.

The chelating chemicals used in the studies and the stability constants of the iron chelates are listed below:

Chelating compound	Stability constant
1. Ferric nitrilotriacetic acid (FeNTA)	10^{15}
2. Ferric N-hydroxyethylenediaminetriacetic acid (FeHEEDTA)	10^{21}
3. Ferric ethylenediaminetetraacetic acid (FeEDTA)	10^{25}
4. Ferric diethylenetriaminepentaacetic acid (FeDTPA)	10^{28}
5. Ferric cyclohexanediaminetetraacetic acid (FeCHDTA)	10^{29}
6. Ferric ethylenebishydroxyphenylglycine (FeEHPG)	$>10^{30}$

Results

► Absorption of FeEHPG From Nutrient Solutions: EHPG is a particularly useful chelating agent for experimental work on uptake of metal and ligand because of its optical activity. The ligand alone absorbs light strongly at 276 mμ; the Fe chelate absorbs maximally at 475 mμ and acidified FeEHPG absorbs strongly at 280 mμ. Up to 10 ppm the absorbancy follows Beer's Law. Thus it is possible to determine the amount of uptake of iron and ligand or ligand alone by plant roots by measuring spectrally the concentrations of FeEHPG and EHPG in nutrient solution. In our studies we used a Beckman Model DK spectrophotometer for this purpose.

By use of the above described method, the differential absorption of Fe from FeEHPG has been reported for sunflowers, zinnias, and soybeans (3, 5). In both studies large quantities of iron were found in xylem exudate following the placement of roots from decapitated plants in nutrient solutions containing FeEHPG. Relatively little loss of ligand from the nutrient solution was demonstrated in these experiments (3, 5).

The present studies on differential uptake of Fe from FeEHPG by soybeans differ from this previous

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work (3, 4, 5) in that intact plants were used instead of decapitated ones.

In initial experiments, the chlorotic soybean plants (4 per container) were supplied with FeEHPG at concentrations of 0.75 and 1.50 × 10^{-5} M. Three replications were used. Loss from the solution as a result of uptake by plants of either iron or ligand from solutions containing these relatively large concentrations of chelated iron was too slight for conclusions to be drawn. Consequently, the experimental procedure was changed in two ways: first, the cotyledons were removed from the young seedlings at the time of transplanting to the pots to reduce the supply of endogenous iron and, second, FeEHPG was supplied at the lower concentration of approximately 0.40 × 10^{-5} M. As in initial experiments, four plants were grown in each container and three replications were used. Controls consisted of similar containers with aerated nutrient solutions containing FeEHPG or EHPG but without plants. The nutrient solutions were sampled at weekly intervals and spectrophotometric determinations of FeEHPG and EHPG made. Prior to sampling, the water levels in the containers were restored to their original heights and the solutions stirred. The results of a typical experiment are shown in table I.

Table I

Absorption of Iron From FeEHPG by Soybean Plants in Nutrient Solutions Over a 6-Week Period

Date of measurement	Conc in M × 10^{-5}				
	Soybean solution		No plant Control solution		No plant control
	Fe	EHPG	Fe	EHPG	EHPG only
June 13	0.39	0.43	0.38	0.42	0.19
June 24	0.35	0.41	0.38	0.46	0.20
July 1	0.27	0.38	0.36	0.46	0.19
July 8	0.24	0.35	0.35	0.48	0.19
July 15	0.19	0.35	0.33	0.42	0.19

Approximately half of the iron disappeared from the nutrient solutions during the 32-day period of measurement, whereas only a small percentage of the ligand disappeared. These results essentially confirm the work of Tiffin et al. (5) on differential uptake of Fe from FeEHPG.

► Effect of Excess Ligand in Nutrient Solution on Growth of Iron-Deficient Soybean Plants: Although the above results indicate strongly that the iron was absorbed preferentially through the root membranes, certain observations on regrowth in other experiments are difficult to explain on the basis of lack of absorption of the ligand. We have had occasion to supply six different iron chelates at low concentrations of iron with varying quantities of ligand in the nutrient solutions to chlorotic PI-54619-5-1 soybean plants. The iron was supplied at either 0.89 or 1.78 × 10^{-5} M and ligands (NTA, HEEDTA, FDTA, DTPA, CHDTA, & EHPG) were given at concentrations of 0.000, 0.262, 0.524, 1.048, 2.096, and 4.192 × 10^{-5} M in these solutions. Three replications of two plants per pot were used for each treatment. Responses of the plants after 14 days were most striking (fig 1) both from the standpoints of regreening (table II) and root development (table III).

Table II

Effect on Regreening of Soybean Plants Supplied Iron at Constant Concentration & Ligand at Varying Concentrations in Nutrient Solutions

Ligand	Conc of ligand in M × 10^{-5}					
	0.0	0.262	0.524	1.048	2.096	4.192
	No. plants that regreened when supplied Fe at 0.89 × 10^{-5} M					
NTA	0*	6	6	6	6	6
HEEDTA	0	4	6	6	6	4
EDTA	0	4	6	6	6	6
DTPA	0	3	6	6	3	0
CHDTA	0	2	4	3	3	0
EHPG	0	2	6	6	2	0
	No. plants that regreened when supplied Fe at 1.78 × 10^{-5} M					
NTA	0	6	6	6	6	6
HEEDTA	0	4	6	6	6	6
EDTA	0	5	6	6	6	6
DTPA	0	6	6	6	4	2
CHDTA	0	0	6	6	3	0
EHPG	0	3	6	6	4	0

* Six plants were used in each treatment.

The striking effect on growth of the relative binding tenacity of iron is shown in figure 1. As the stability constant of the Fe chelate increased from 10^{15} (FeNTA) to 10^{29} (FeCHDTA), there was a continuous reduction in total growth of the plants. This reduction in growth was independent of the interactions between iron and ligand ratios. It was noted that where the higher concentration of iron was used, this gradient of response was less apparent.

Where weaker chelates (FeNTA, FeHEEDTA, & FeEDTA) were used, the plants regreened rapidly and completely, seemingly regardless of concentration of iron or ligand supplied. This was not the case, however, for plants grown in nutrient solutions containing FeDTPA, FeCHDTA, or FeEHPG. Plants given these stronger iron chelates (stability constants range from 10^{28} to $>10^{30}$) usually showed several responses in common. First, the degree of regreening of the plants was correlated with the level of iron supplied. Second, the iron chelates were not effective in promoting regreening at the lowest concentration used. The only exception to this was FeDTPA. This chelate differs from the others in that it carries two negative charges instead of one. Possibly absorption of Fe by plants from this compound is less difficult than from chelates having similar binding powers but which carry only one negative charge. And, third, regreening was hampered by use of ligand in excess of that required

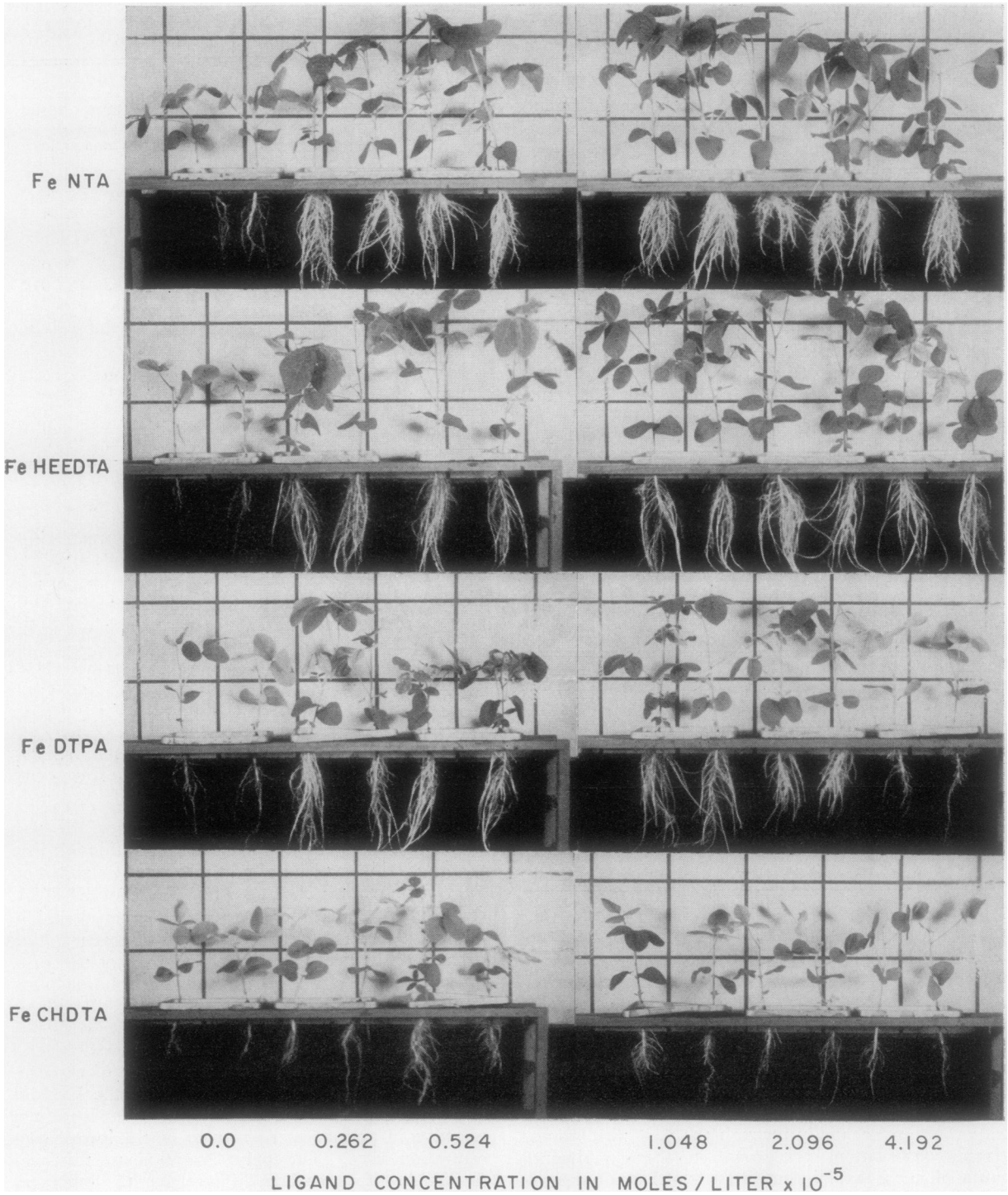

Fig. 1. Effect of verying levels of ligands on growth, regreening, and root development of soybean plants. Iron was supplied at 0.89 $\times$ 10^{-5} M in all cases. Note that as the stability constant of the iron chelate increases (FeNTA $\rightarrow$FeCHDTA) the growth is reduced regardless of either iron or ligand concentration.

Table III

Effect on Root Growth of Soybean Plants Supplied Iron at Constant Concentration & Ligand at Varying Concentrations in Nutrient Solutions

Ligand	Conc of ligand in M × 10^{-5}					
	0.0	0.262	0.524	1.048	2.096	2.192
	No. of plants that developed good root systems when supplied Fe at 0.89 × 10^{-5} M for 14 days					
NTA	0*	6	6	6	6	6
HEEDTA	0	6	6	6	6	6
EDTA	0	0	6	6	6	6
DTPA	0	3	6	6	4	0
CHDTA	0	0	3	3	0	0
EHPG	0	0	0	0	0	0
	No. of plants that developed good root systems when supplied Fe *at* 1.78 × 10^{-5} M					
NTA	0	6	6	6	6	6
HEEDTA	0	6	6	6	6	6
EDTA	0	2	6	6	6	6
DTPA	0	6	6	6	6	3
CHDTA	0	0	6	6	4	0
EHPG	0	0	6	3	0	0

* Six plants were used in each treatment.

to chelate all of the iron present in the solution. In fact, plants grown at the highest levels of DTPA, CHDTA, and EHPG almost always became more chlorotic than they were before treatment was started.

The effects on root growth of these various iron chelates were in most ways similar to the effects on regreening (table III). It is apparent that root growth is particularly sensitive to iron supply and that as iron availability increased, whether through the addition of more iron to the solutions or through the use of weaker chelating agents, root growth increased proportionately. As might be expected from the results on regreening, roots generally failed to develop when placed in solutions of strong ligands (DTPA, CHDTA, or EHPG) containing quantities in excess of that required to chelate the iron present.

That these adverse effects on growth were the result of the excess ligand in the solution was borne out by the following experiment. Chlorotic soybean plants were grown in solutions containing 0, 20, 40, 80, 160, and 320 × 10^{-5} M of FeEHPG. In addition, other plants were grown in solutions containing 160 and 320 × 10^{-5} M EHPG without iron. Regreening was rapid and root growth excellent for the plants given 20, 40, and 80 × 10^{-5} M of FeEHPG. At 160 and 320 × 10^{-5} M of FeEHPG the plants turned red within 72 hours of treatment. The plants given 320 × 10^{-5} M of FeEHPG subsequently died. Plants given EHPG but no iron wilted and died within 48 hours of being placed in the nutrient solutions. It must be concluded from these results that FeEHPG is not toxic to plants even at quite high concentrations but when the ligand is supplied without sufficient iron to chelate it, EHPG can be very deleterious.

Tiffin et al. (5) have reported the observation that "colored exudates could be obtained from stems of certain plants when their roots were in FeEDDHA" (FeEHPG). These exudates absorbed light at 480 mμ, thus indicating that FeEHPG was present in the exudate. In our studies, we found that if FeEHPG were supplied in very high concentrations (either to nutrient solutions at pH 6.0 or on a clay soil with a pH of 7.95), colored exudates could be obtained from cut stems and the leaves of such plants turned dark red within 48 hours. Extraction of leaf homogenate with butanol to remove anthocyanins left a pale red liquid which absorbed strongly at 480 mμ and, upon acidification, absorbed strongly at 276 mμ. There seems to be little doubt that FeEHPG did exist in such plants. However, the concentration of FeEHPG required to produce such results makes it highly questionable whether such uptake occurs under more usual circumstances of application.

► Uptake of EHPG From Nutrient Solutions by Soybean Plants: In view of the toxicity of the stronger chelating agents when supplied in amounts greater than that required to chelate all of the iron in the nutrient solution, more experimentation on the uptake of strong ligands such as EHPG, DTPA, and CHDTA seemed advisable. The simplest hypothesis explaining the toxicity symptoms in the soybean plants was that an iron deficiency was caused by uptake of the non-iron chelated ligand. Both the severe chlorosis induced in the foliage and the lack of root development in these plants are typical of iron deficiency symptoms. If the symptoms were an expression of iron deficiency, it was likely that the ligand was being absorbed by the plant. Conceivably, once the ligand was absorbed by the roots it would compete for the iron in the plant. And because of the extreme tenacity with which these ligands bind iron, the net effect would be to rob the plant of its endogenous iron supply.

In order to test this hypothesis the following experiment was carried out. Large PI-54619-5-1 soybean plants with well developed root systems were used as test plants. The tops of the plants were removed so that only the primary leaves and the two lowest lateral buds were left. These plants were then placed (2 per pot) in 1-gallon polyethylene containers. The following treatments were used in the nutrient solutions:

Treatment	M × 10^{-5} FeEHPG	M × 10^{-5} EHPG
1	0.0	0.0
2	0.15	0.0
3	0.15	0.30
4	0.15	0.60
5	0.0	0.30
6	0.0	0.60

Comparable solutions without plants were used as controls. Three replications of each treatment were used. Solutions were sampled spectrally for FeEHPG, Fe, and EHPG content at the beginning

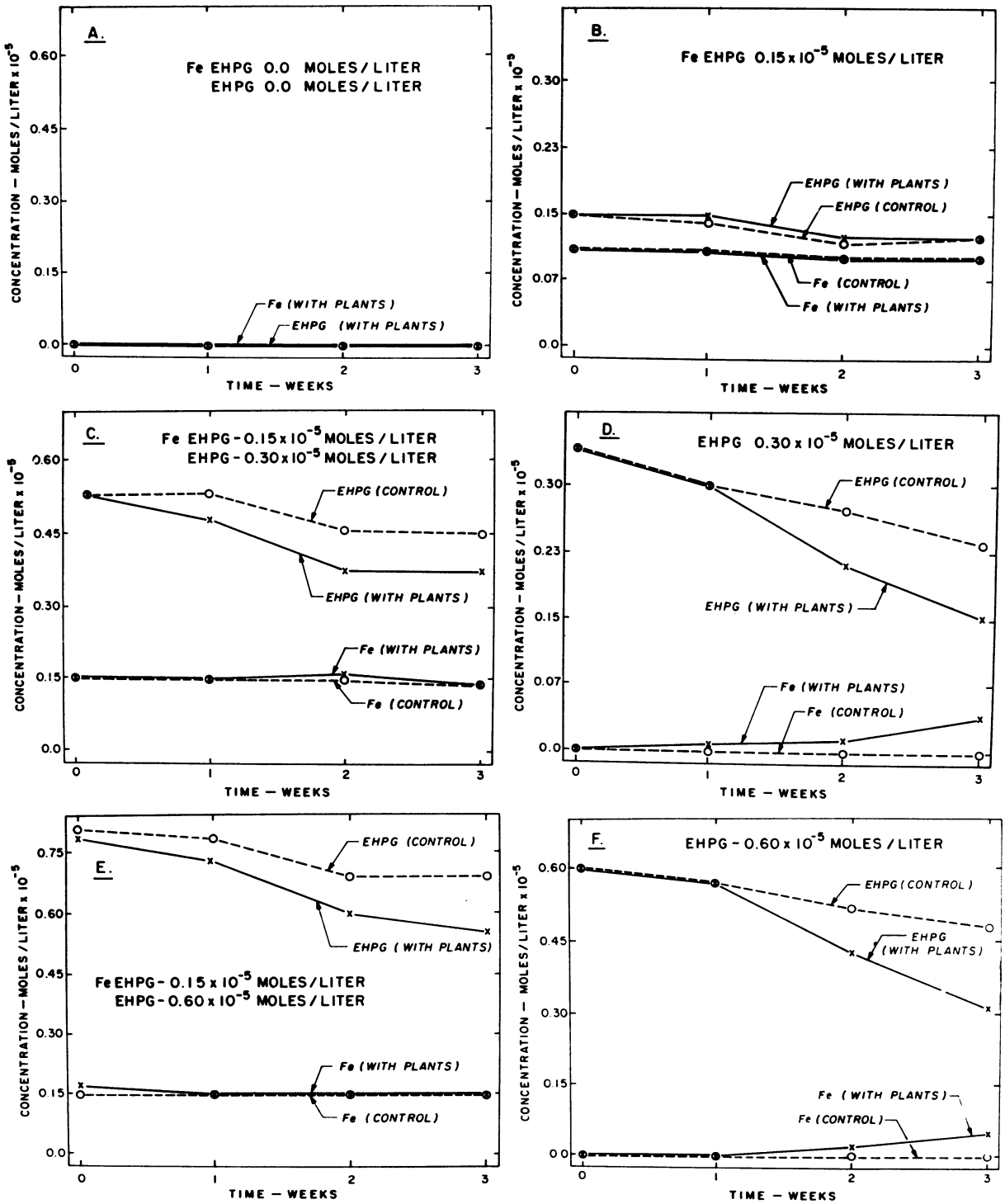

Fig. 2A–F. Changes in concentrations of Fe and EHPG in nutrient solutions in which soybean plants were growing. Control solutions contained no plants but were aerated. Note that EHPG disappeared from the solutions only when supplied in excess of that required to chelate the iron present. FeEHPG was supplied at 0.15 $\times$ 10^{-5} M but the relative chelating capacity of EHPG was such that 0.10 $\times$ 10^{-5} M of FeEHPG was in the solution. Note that where excess ligand was supplied (c & e) the concentration of Fe rose to 0.15 $\times$ 10^{-5} M. The standard deviation of a single determination for EHPG was $\pm$ 0.018 $\times$ 10^{-5} M; for a single determination of Fe, the value is $\pm$ 0.023 $\times$ 10^{-5} M.

of the experiment and weekly for 3 weeks. Before each sampling the water levels were restored to the top of all pots and the solutions mixed thoroughly, figure 2.

It is apparent that where iron and EHPG were supplied at about equimolar concentrations (fig 2b) no change in concentration of either component was detected. In fact, iron uptake through plant roots was not demonstrated in any of the treatments (figs 2b, c, e). However, where the concentration of ligand was in considerable excess of iron concentration, there was a substantial decrease in ligand concentration in the nutrient solutions (figs 2c, d, e, f) during the 3-week period of growth. Uptake of ligand was greater where 0.60×10^{-5} M was supplied than where 0.30×10^{-5} M was used. It is significant that where ligand without iron was supplied (figs 2e & 2f) a small but demonstrable amount of FeEHPG was present in the nutrient solutions at the end of the growth period. The ligand had undoubtedly extracted the iron from the roots of the plants. Competition between strong chelating agents and endogenous iron in roots has recently been reported by Brown et al. (1).

Table IV

Effects on Regrowth of Soybean Plants in Nutrient Solutions Containing Various Levels of FeEHPG & EHPG

Treatment** conc in M $\times 10^{-5}$	Type of foliage growth		
	No. of green	No. of mildly chlorotic	No. of very chlorotic
1. 0.10 FeEHPG*	4	2	0
2. 0.15 FeEHPG plus 0.30 EHPG	6	0	0
3. 0.15 FeEHPG plus 0.60 EHPG	0	6	0
4. No iron or ligand	0	5	1
5. 0.30 EHPG	0	2	4
6. 0.60 EHPG	0	0	6

* FeEHPG was added at a concentration of 0.15×10^{-5} M but the relative chelating capacity of EHPG was such that 0.10×10^{-5} M of FeEHPG was in the solution. Note that when excess ligand was supplied (treatments 3 & 4) the concentration of Fe was 0.15×10^{-5} M.

** Six plants were used in each treatment.

Growth responses of the plants were very striking (table IV). In general, regreening was related to iron concentration in the solutions, but the presence of EHPG in a non-iron chelated form also influenced plant response. Plants supplied with neither iron nor ligand had slightly chlorotic regrowth, but those given no iron but either 0.30 or 0.60×10^{-5} M EHPG produced extremely chlorotic regrowth. Plants given 0.15×10^{-5} M FeEHPG or 0.15×10^{-5} M FeEHPG plus 0.30×10^{-5} M EHPG grew well and were not chlorotic. Those given 0.15×10^{-5} M FeEHPG plus 0.60×10^{-5} M EHPG grew well but were slightly chlorotic in appearance.

If the very chlorotic appearance of the plants supplied EHPG but no iron was caused by iron deficiency, it should be possible to overcome this chlorosis by spraying the leaves with inorganic iron. This was done on one of the two plants in each pot where treatments were supplied neither iron nor EHPG and those given 0.30 or 0.60×10^{-5} M EHPG, respectively. Iron was applied as a ferrous sulfate spray (0.1 g $FeSO_4$/100 cc distilled H_2O) twice during a 10-day period. At the end of this time the unsprayed plants were still chlorotic, whereas the sprayed plants all developed dark green leaves. It would appear that the acute chlorosis induced by growing the plants in nutrient solutions lacking iron but containing EHPG was a result of the EHPG being taken up by the plant and chelating iron already in the plant. This resulted in iron becoming unavailable to the plant and typical iron deficiency symptoms ensued.

The most surprising observation made following the regreening of the $FeSO_4$-sprayed plants concerned a color change in the nutrient solutions that contained EHPG. These solutions turned pink during the 10-day period. On the 10th day, the solutions contained iron at the levels of 0.075×10^{-5} M FeEHPG in the pots that had originally contained no iron plus 0.30×10^{-5} M EHPG and iron at a concentration of 0.12×10^{-5} M FeEHPG in the pots that originally contained no iron plus 0.60×10^{-5} M EHPG. It would appear that some of the $FeSO_4$ sprayed on the leaves had moved down into the roots and out into the nutrient solutions where it was chelated into FeEHPG.

Discussion

The results of Tiffin and Brown and co-workers indicated that when strongly chelated iron was supplied to decapitated plants in nutrient solutions during a 22-hour period, the iron was preferentially absorbed by roots with most (but probably not all) of the ligand remaining in the solution. The work described here substantiates their results. The results of the present study further suggest that when a ligand is supplied in excess of that required to chelate iron in the solution, and hence chelates weakly with other cations—such as calcium and magnesium—in the nutrient solution, the ligand (chelate ?) is readily taken up through plant roots.

It is not known whether these weakly bound chelates are split at the root membrane (as is the case for iron chelates) or whether the whole chelate molecule is taken up by the plant. It would not be surprising if an analogous situation existed, but the possibility of absorption of the entire chelate cannot be eliminated.

These results also suggest that the ligands which bind iron most strongly on their entrance into the plant also bind endogenous iron so that it is no longer available for normal metabolic processes. Iron probably exists in plants in the form of chelates. It is interesting to note that when non-iron chelated ligands were present in the solutions, the PI-54619-5-1

soybean plants were able to grow well so long as the stability constant of the iron chelates of these ligands was 10^{25} or lower (FeNTA, FeHEEDTA, & FeEDTA). Apparently, the concentration of iron in the plant, the concentration of the natural chelates and the stability constant of the natural chelates or the constant alone were all such that competition for the endogenous iron supply by the synthetic ligands was not sufficient to cause symptoms of iron deficiency to appear. Adverse effects on regrowth occurred when strong iron-chelating ligands (DTPA, CHDTA, & EHPG) were supplied but usually only when their concentration exceeded the iron concentration in the solutions. Since non-iron chelated EHPG was readily taken out of the nutrient solution by soybean plants, it seems probable, in view of the similar growth effects caused by DTPA and CHDTA, that these ligands are also taken up by the plant. And, if this hypothesis is true, then all of the ligands can be taken up. By working with different plant species and a series of chelating agents, more information could probably be gained on this point. Tobacco plants have recently been shown to possess a natural chelating agent with a pK of between 10^{-17} + 10^{-21} (2).

It might be argued that some of the EHPG disappeared from the nutrient solution because of a breakdown of the compound through the activity of root exudates and that the remaining EHPG pulled enough iron from the plants to cause iron deficiency symptoms to appear. However, such a hypothesis cannot easily be reconciled with the observations that when EHPG was chelated with iron, very little of the ligand disappeared during several weeks of exposure to plant roots, and the appearance of iron in the solutions which contained EHPG (but no iron) was slight and appeared long after severe chlorosis deficiency symptoms had been induced.

The fact that plants can tolerate large concentrations of a strongly bound chelated iron without adverse effect points out the criticality of iron concentration in the plant and the nutrient solution or the solution alone. It appears that these strong ligands act as competitive inhibitors in iron metabolism and it is only when the available iron supply in the plant is borderline that strong chelating agents will cause iron deficiency symptoms. So long as strong ligands are chelated with iron, uptake of iron by the plant appears to be limited to such an extent that insufficient ligand is freed for the plant to be able to absorb the ligand in harmful amounts.

Summary

Absorption of Fe from ferric ethylene bishydroxy phenyl glycine (FeEHPG) in nutrient solutions was demonstrated in intact PI-54619-5-1 soybean plants. EHPG was taken up through roots only when its concentration exceeded that of the iron in the solutions.

The severe chlorosis that resulted from growing plants in solutions containing low levels of EHPG and no iron or solutions containing large excesses of EHPG, cyclohexanediaminetetraacetic acid (CHDTA), or diethylenetriaminepentaacetic acid (DTPA) over iron suggests that all of these ligands (when unchelated with iron) are picked up by the plant roots where they chelate iron which is in the plant. Spraying leaves of such plants with $FeSO_4$ resulted in a rapid regreening and, in the case of EHPG, a substantial amount of iron was moved out of these plants and into the nutrient solution.

It appears that natural chelates in PI-54619-5-1 soybean plants can compete favorably for iron with synthetic chelates having stability constants of 10^{25} or less [ferric nitrilotriacetic acid (FeNTA); ferric N-hydroxyethylenediaminetriacetic acid, (FeHEEDTA); & ferric ethylenediaminetetraacetic acid, (FeEDTA)] but not with strongly bound chelates (FeDTPA, FeCHDTA, or FeEHPG), all of which have stability constants $>10^{28}$. It is suggested that the principal reasons that EHPG from FeEHPG does not enter plant roots more easily are: A, that the iron is so strongly bound to the ligand that the plant is not able to split the FeEHPG molecule and differentially take off the iron in large enough quantities to make ligand available for chelation with other metals in the nutrient solution, and B, rate of uptake of non-iron chelated ligand is slower than rate of uptake of iron by iron-deficient plants.

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